博 士 学 位 論 文

Identification and Physiological Activities of Organic Compounds Produced from Natural Product Resources, and Effective Utilization of the Resources

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PREFACE

The work in this thesis was conducted under the guidance of Professor Masato Nomura at the Department of Natural Products Chemistry of Kinki University from 2013 to 2016.

The inherent function of natural products is to maintain ecological balance. Advances in technology have allowed the extraction and separation of these resources and have allowed for their structures to be elucidated. These advances have presented the opportunity to use the active ingredient from plant resources in their pure form. In the 21st century, this has led to the discovery of new bioactive natural products and has stimulated drug development and advances in health maintenance and the treatment of disease. In recent years, degradation of the environment and changes in lifestyle has contributed to the emergence of new diseases. Consequently, new pharmaceutical products and functional materials are necessary. In this respect, finding new plant resources and assessing their functions is important.

This study aims to evaluate the physiological activity of organic compounds from natural product resources and to assess their usefulness to engineering and industry. I shall be happy if this work contributes in some way to natural products chemistry.

Shuhsien Wu

Natural Products Chemistry, Kinki University

2016

LIST OF PUBLICATIONS

Contents in thesis are published in following papers.

1. Physiological Activity of Chinese Lichen (Gyrophora esculenta) Component, Methyl 2,4-Dihydroxy-6-methylbezoate and the Related Compounds

   Shuhsien Wu, Zhendong Zhao, Yoshiharu Okada, Yoshiyuki Watanaba, Toshiyuki Takahata, Toshio Inoue, Eiji Otsubo, Jing Wang, Yanju Lu and Masato Nomura

   Asian Journal of Chemistry, **26** (3), 702-708, 2014.

2. Evaluation of the Fatty Acid Composition of the Seeds of Mangifera indica L. and Their Application

   Shuhsien Wu, Megumi Tokuda, Ayaka Kashiwagi, Atsushi Henmi, Yoshuiharu Okada, Shinya Tachibana and Masato Nomura

   Journal of Oleo Science, **64** (5), 479-484, 2015.

3. Deodorizing effect of mango seed kernel oil for 10 odorous substances

   Shuhsien Wu, Atsushi Henmi, Shinya Tachibana and Masato Nomura

   Research reports of the Faculty of Engineering, Kinki University, **49**, 1-6, 2015.

4. Novel physiological roles of mango seed oil using proteomic analysis of differentially expressed proteins

   Shuhsien Wu, Kenichi Matsumoto, Shinya Tachibana, Toshio Inoue and Masato Nomura

   Pharmacometrics, **88** (3/4), 57-65, 2015.

CONTENTS

INTRODUCTION

In ancient China, the legend of Shennong avers that the emperor tasted hundreds of herbs to distinguish medicinal varieties; in so doing, he initiated the study of pharmaceuticals. Plants have long occupied a mystical and functional role in human society. A famous Japanese tale, *The Hare of Inaba* (Kojiki version), conveys how the god of medicine healed a hare's wound using the ear of reedmaces. During the Dragon Boat Festival (May 5) in China, calamus or mugwort is customarily hung up for the prevention of disease. Egypt is considered to be the origin of western medicine and pharmacy. Aromatic plants were incorporated into daily life, notably for preserving dead bodies [1)] and blending a cosmetic perfume known as *kyphi* [2)]. In the latter half of the 18th century, the Swedish chemist Carl Wilhelm Scheele began separating organic acids from plants [3)]. The extraction, isolation, and structural elucidation of natural organic compounds progressed rapidly due to improved analytical instruments and measurement techniques.

Plants have been important resources for preserving the global environment. The advance of civilization has influenced the environment; conversely, the environment has affected civilization and necessitated a reconsideration of human values. Plants provide the food, clothing, and shelter required for humans and animals, and they serve a vital role in daily life and culture.

Plant-based therapies have a well-established history. *Forest bathing* has been observed to mitigate stress by inducing relaxation [4-6)]. Forest trees and flowers co-evolved naturally over time. Monoterpenoids (16 hydrogen atoms with 10 carbon atoms) are a class of volatile compounds present in various forest plants; they have been used as relaxants, antibacterials [7)], insect repellants [8)], and antioxidants [9)]. Dr. Boris P. Tokin coined the word *phytoncide* in 1928 to describe a volatile substance that plants exude to kill competing organisms. Plants deodorize the forest [10)] by neutralizing malodorous compounds or by masking the odors with other compounds.

Lipids are a significant disease factor due to life styles that involve drinking, smoking, lack of exercise, and social stress [11)]. Lipids store energy *in vivo*; they feature as components of the cell membrane and are biosynthesized into physiologically active precursors like prostaglandin [12)]. Commercially, lipids are used in cosmetics and pharmaceuticals [13, 14)]. Fatty acids,

triacylglycerol, phospholipids, and cholesterol are mainly introduced into humans from ingested food.

Diseases related to diet and lifestyle (high blood pressure, diabetes, and obesity) have been increasing in frequency [15]. Hyperlipidemia, excess levels of lipids in the bloodstream, occurs when the biological metabolism of lipids is insufficient [16]. For example, arteriosclerosis may be caused by the excess consumption of cholesterol: the concentration of LDL increases in the bloodstream, cholesterol accumulates on the vessel walls, and blood flow decreases. Lipid signaling molecules have also been implicated in cell proliferation, differentiation, and immune system activity [17]. Foods that contain chemical functionality have gained attention for preventative and therapeutic treatments.

Foods have been classified according to function: nutrition (primary), sense (second), and biological regulation (third) [18]. The functional components in food have been related to immunity [19], antitumor behavior [20], suppression of blood glucose and blood pressure [21], and antioxidant effects [22]. The oral administration of evening primrose oil, a lipid that contains γ-linolenic acid, was shown to be effective in treating atopic dermatitis [23]. Low-grade autoimmune disorders and chronic inflammatory disease have been observed in Eskimo populations, due to the excessive intake of eicosapentaenoic acid (EPA) [24, 25]. β-carotenoid, biosynthesized from the isoprenoid pathway, is a secondary metabolite that has been reported to inhibit the proliferation of colon cancer cells [26].

Oxidative stress is caused by the excess production of active oxygen and oxygen free radicals by lipid peroxidation. These free radicals damage proteins, enzymes, and nucleic acids; they are related to aging and disease. Antioxidants are important because they counter the effects of free radicals in the body. Antioxidants are found in natural foods and fermentation products [27], including enzymes (superoxide dismutase, catalase), vitamins (vitamin E, ubiquinol), fat-soluble low–molecular weight molecules (bilirubin), and water-soluble molecules (glutathione).

Plants have been present on earth for over 250,000 years. Many of the biochemical mechanisms that they have evolved remain unexplained; however, various techniques for

evaluating physiological activity (antioxidation [28], antiallergenic behavior [29], and immunomodulation [30] have been discovered. In recent years, the influence of the environment and lifestyle has contributed to the emergence of new diseases. Research has exploited the inherent functions of plant-based compounds to solve human-health problems.

This paper examines the potential commercial uses of selected plants. Rock tripe (*Gyrophora esculenta*) belongs to the umbilicariaceae family and is the subject of Chapter 1. The samples for this study were grown on the sandstone cliffs at Zhangjiajie, Hunan province, China. The active components were identified, and the physiological activity of the natural and synthetic compounds was investigated. The seed of mango fruit (*Mangifera indica* L.) is usually discarded; however, Chapter 2 explores its use as a value-added commodity chemical. Mango seed oil (MSO) samples from Miyazaki, Japan and Taiwan were compared; their performance was assayed for use in cosmetics, deodorants, and detergents. The antitumor activity of MSO was examined for HeLa cells.

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CHAPTER 1 Chinese lichen: the physiological activity of methyl 2,4-dihydroxy-6-methylbenzoate and its derivatives

1. INTRODUCION

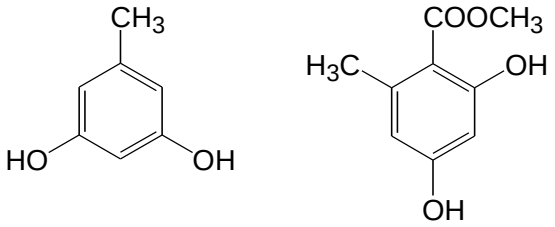
In modern society, stress has been cited as one of the main obstacles to mental and physical health [1)]. Lifestyle-related diseases (cancer, hardening of the arteries, hypertension, diabetes, etc.) are mainly caused by active oxygen and free radicals [2, 3)], which are produced in excess in the body. They induce various diseases by damaging cells through protein denaturation, lipoperoxidation, nucleolytic degradation, and enzyme inactivation [4, 5)]. Recent trends in health-consciousness have promoted the consumption of traditional foods; these contain beneficial active compounds linked to the prevention of disease or antiaging [6)]. Lichen has been consumed by humans since antiquity; its unique composition (it is an homobium of fungi and algae) is believed to confer medicinal qualities [7, 8)]. Lichen has been reported to effectively inhibit lifestyle-related diseases, such as hypertension and hypercholesteremia [9)].

Gyrophora esculenta, also known as *Umbilicaria esculenta*, (Miyoshi) minks, and iwatake, grows on the surface of rocks or on tree trunks; it is distributed widely from the extratropical belt of eastern Asia (including China, Japan and Korea) to mountainous areas such as the Himalayas. Members of the family *Umbilicariaceae* look like rounded, thin leaves: they have a gray top surface, black back surface, and thick spines. *Umbilicariaceae* grow slowly at a rate of 1 cm every two or three years [7, 8, 10)]. Hashimoto *et al.* reported that iwatake exhibits physiological activity as an anticancer agent, anti-inflammatory, and glycuresis prophylactic [11, 12)]. This study identified and investigated an antioxidant compound contained in the oily extract of Chinese lichens *Gyrophora esculenta* (Miyoshi). Several analogous chemicals were derived from the natural compound and likewise investigated. The physiological activity of the compounds differs based on their chemical substituents. The effects of these compounds on antioxidation, cytotoxicity, the suppression of cytokine production, and the inhibition of histamine release are reported herein.

2. RESULTS AND DISCUSSION

2.1. Active compounds and their activities

The crude oil was obtained from a methanol extraction of the Chinese lichens and analysis of the composition of the oil was carried out with gas chromatography mass spectroscopy (GC-MS). The results are shown in Fig. 1. 3,5-Dihydroxytoluene (orcinol) (**1**) and methyl 2,4-dihydroxy-6-methylbenzoate (methyl orsellinate) (**2**) are identified as the primary components (GLC%, 44:56). The crude oil was extracted sequentially with hexane, diethyl ether, and ethyl acetate. The antioxidant activities of these fractions were assessed on the basis of a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and a superoxide dismutase-like (SOD-like) assay. The results are shown in Table 1.



(**1**) (**2**)

Fig. 1 Structure of compounds (**1**) and (**2**).

Table 1 DPPH radical scavenging assay and SOD-like assay of *Gyrophora esculenta*.

Extract [a]	DPPH Radical Scavenging Assay		SOD-like Activity Assay	
	Scavenging Rate (%) [b]	SC_{50} [c]	Inhibition Rate (%)	
			0.1 mg / mL	1 mg / mL
Methanol	35.9	370.9	22.2	23.5
Hexane	25.4	>400	9.7	10.7
Diethyl ether	63.3	113.7	22.7	23.9
Ethyl acetate	84.5	43.9	18.8	29.5
Residue	23.0	>400	8.7	9.3
α-Tocopherol [d]	100.0	6.0	—	—
Ascorbic acid	—	—	12.6	88.9

a) Concentration : 1 mg / mL b) Corrected concentration : 0.2 mg / mL

c) 50% Scavenging Concentration (mg / mL) d) Concentration : 1 mM

The ethyl acetate fraction shows the highest DPPH radical scavenging activity, and the diethyl ether and ethyl acetate fractions show high activity in the SOD-like assay. At low concentration (0.1 mg/mL), the diethyl ether and ethyl acetate fractions show higher SOD-like activities (22.7% and 18.8%, respectively) than that of ascorbic acid (12.6%). The diethyl ether fraction was chromatographed on silica gel to separate the effective antioxidant compounds. As shown in Table 2, fraction (A-4) shows the highest

inhibition of the four fractions (A-1)–(A-4), equal to that of ascorbic acid (12.6%) in the SOD-like assay. In addition, fraction (A-4) was re-chromatographed on silica gel to give pure methyl orsellinate (**2**) as a white crystalline solid. Its active oxygen inhibition was determined at 0.1 and 1.0 mg/mL and was found to increase from 11.1% to 14.9%, respectively. Therefore, compound **2**, an important component identified in the methanol extract of Chinese lichens, has great potential as an antioxidative agent.

Table 2 SOD-like assay for fractions (A-1)-(A4-5).

Fraction	SOD-like Activity Assay	
	Inhibition Rate (%)	
	0.1 mg / mL	1 mg / mL
A-1	17.7	24.7
A-2	18.1	24.5
A-3	16.0	26.0
A-4	23.3	26.0
A4-1	2.4	2.4
A4-2	11.1	14.9
A4-3	11.6	12.5
A4-4	6.5	16.1
A4-5	9.9	15.8
Ascorbic acid	12.6	98.7

2.2. Synthesis and activity of compounds related to (**2**)

Following the results for the physiological activities of methyl orsellinate (**2**), derivatives of **2** were synthesized, as shown in Scheme 1. Four compounds, methyl 2,4-dihydroxybenzoate (**4**), methyl 2-hydroxy-4-methoxybenzoate (**5**), methyl 2,4-dimethoxybenzoate (**6**), and methyl 4-hydroxy-2-methoxy-benzoate (**7**), were synthesized from 2,4-Dihydroxybenzoic acid (**3**). As discussed previously, the physiological activities of compound **2** and its derivatives, such as DPPH radical scavenging effects, antitumor activity, and antibacterial activity, have been reported [13-15]. In this chapter, the antioxidant activities of compound **2** and its derivatives evaluated on the basis of SOD-like assays at different concentrations are reported. The results are shown in Table 3. Compounds **4** and **7** were exhibited medium inhibition activities (10.9%–15.8%) at a concentration of 1.0 mg/mL. Furthermore, at a concentration of 0.1 mg/mL, compound **7** shows a 9.5% inhibition rate, which is close to that of ascorbic acid (10.2%) as a reference compound.

Scheme 1

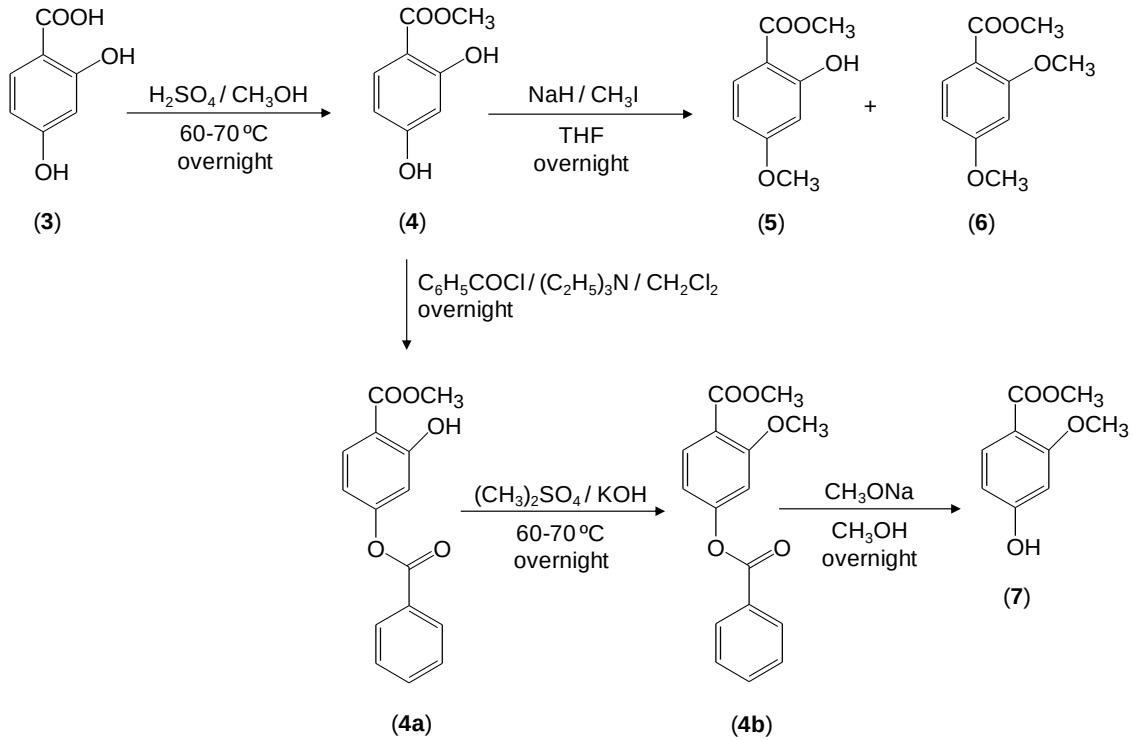


Table 3 SOD-like assay for four compounds (4-7).

Compound	Inhibition rate (%)	
	0.1 [a)]	1.0
4	2.7	15.8
5	4.4	6.5
6	1.0	1.5
7	9.5	10.9
Ascorbic acid	10.2	97.1

a) Concentration (mg/mL)

These results suggest that the hydroxyl group at the *para* position of the aromatic ring plays an important role in antioxidant activity. My proposed mechanism for the antioxidant activity of compound **2** is shown in Fig. 2. The hydroxyl group of compound **2** reacts with the active oxygen, and the phenoxy radical formed is transformed to an oxy radical.

The structure of compound **2** changes into a *para* quinoid structure [16)]. The active radical is stabilized via the resonance effect. Since the hydroxyl group at the *ortho* position of compound **2** forms a hydrogen bond to the ester carbonyl group, the hydroxyl group slightly influences the antioxidant activity.

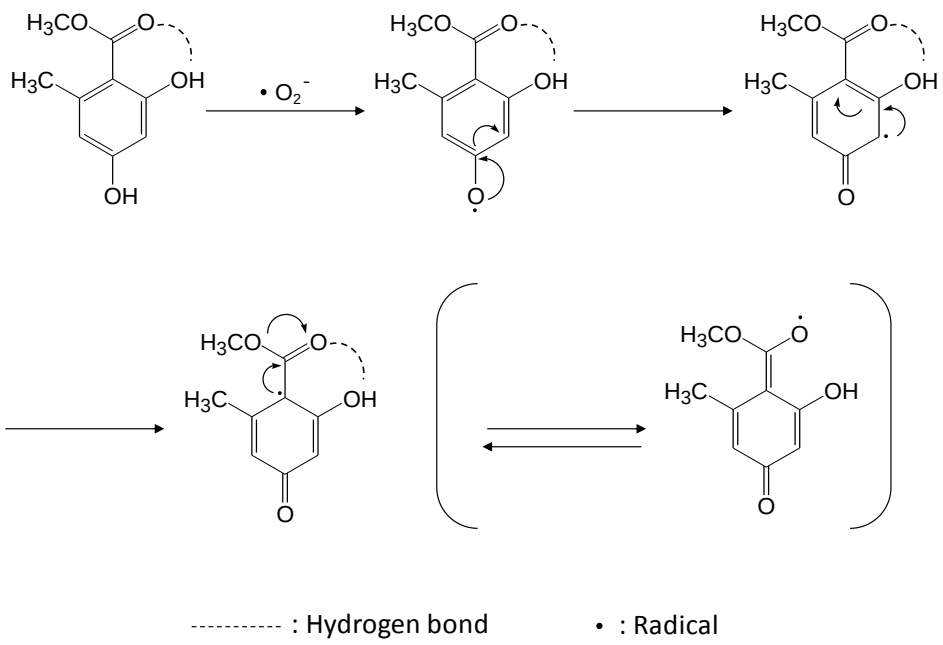


Fig. 2 Plausible structure of methyl-2,4-dihydroxy-6-methylbenzoate radical generated by active oygen.

Next, the antioxidant capacity of compounds **2** and **4**–**7** were assessed using a biological antioxidant potential (BAP) test kit. The capacities of ascorbic acid and α-tocopherol are shown in Table 4, which were determined at different concentrations (0.1–1.0 mg/mL) to calculate their equivalent amounts as standards.

Table 4 BAP of ascorbic acid and α-tocopherol.

Compound	Antioxidant potential (μm)				
	0.01 [a)]	0.1	0.3	0.6	1.0
Asocorbic acid	785	1335	2241	4115	6412
α-Tocopherol	780	1012	1368	2237	3148

a) Concentration (mg/mL)

The BAP value of **7** is 1119 μM, which is the same antioxidant activity as that of a 0.07 mg/mL solution of ascorbic acid or a 0.15 mg/mL solution of α-tocopherol (Table 5). While compound **2**, the main component in fraction A4-2, exhibits high SOD-like activity, the BAP value of compound **2** is only 752 mM. The BAP values of compounds **4**, **5**, and **6** are also below 890 μM. These results indicate that a structure bearing a hydroxyl group at the *para* position with no hydrogen bonded methoxy group at the *ortho* position is important for antioxidant activity. Therefore, compound **7** shows high antioxidant activity.

Table 5 BAP value and standard compound equivalent concentration 2, 4-7.

Sample	BAP value (μM)	α-Tocopherol (mg/mL)	Ascorbic acid (mg/mL)
2	752	0.006	0.011
4	726	N.D. [a)]	0.006
5	887	0.062	0.035
6	830	0.038	0.025
7	1119	0.158	0.075

a) N.D. : Not detect

Table 6 A549 cell toxicity for compounds (**2**) and (**4-7**).

Concentration (μm)	IL-8 production, the percentage of control				
	Compound				
	2	4	5	6	7
1	103	97	110	101	93
10	103	90	110	103	91
100	97	86	129	104	88

According to the immunofluorescent method [17)], cytotoxicity testing of compounds **2** and **4**–**7** was performed with A549 cells from an alveolar epithelial cell line derived from humans. As shown in Table 6, the cell survival rates for all compounds are higher than 80%, which indicates a lack of cytotoxicity. At a concentration of 100 μM, the cell survival rates for compounds **4** and **7** are 86% and 88%, respectively. The hydroxyl group at the *para* position, which was shown earlier to affect the antioxidant activity, also affects cell cytotoxicity. In contrast, the cell survival rate for compound **2** is very high (97%) at the same concentration. Therefore, the toxicity of a compound bearing a hydroxyl group at the *para* position is mitigated when a methyl group is located at the *ortho* position.

Alveolar epithelial cells are known to produce IL-8, an inflammatory cytokine, in response to oxidative stress. Consequently, anti-inflammation activity tests on A549 cells were performed with an enzyme-linked immunosorbent assay (ELISA) kit. The results are summarized in Table 7. Although the IL-8 production rates for all compounds are more than 80%, which indicates no significant inhibitory effect, compound **2** shows slightly lower rates regardless of the concentration. Thus, the presence of the hydroxyl group at the *para* position also seems to affect the production of cytokines.

Table 7 Anti-inflammation activity for compounds (**2**) and (**4-7**).

Concentration (μm)	IL-8 production, the percentage of control				
	Compound				
	2	4	5	6	7
1	92	107	112	88	104
10	93	101	104	117	100
100	91	101	110	94	95

The antiallergenic properties of compounds **2**–**7** were assessed on the basis of a histamine-release assay. An itch-inducing agent (compound 48/80) [18], which is known to cause degranulation of rat peritoneal mast cells, was added to the cells, and the released histamine contents were determined using an amino acid analyzer. The results are summarized in Table 8. Compared with a control, compounds **2** and **4** show histamine-release inhibitory effects of 24.21% ± 2.08% and 21.08% ± 3.76%, respectively. These results indicate that hydroxyl groups at both the *ortho* and *para* positions are important for histamine-release inhibition.

Table 8 Anti-histamine-releasing of compounds (**2**) and (**4-7**).

Compound *	Histamine release (%)
Control	35.23 ± 4.53
2	24.21 ± 2.08 **
4	21.08 ± 3.76 **
5	34.42 ± 5.14
6	40.01 ± 3.71
7	33.42 ± 8.27

* Concentration (100 μg/mL).

** $p < 0.05$ compared with the control group (Dunnett's test).

Furthermore, the dependence of histamine-release suppression on sample concentration was investigated. As shown in Table 9, histamine-release suppression is significant at a concentration of 30 μg/mL. The structures of compounds **2** and **4** are similar to those of quercetin and resveratrol [19, 20], which are well-known antihistamines. The hydroxyl groups at the *ortho* and/or *para* positions of compounds **2** and **4** are considered to be involved in the suppression of histamine release [21, 22].

Table 9 Anti-histamine-releasing of compounds (**2**) and (**4**).

Compound *	Histamine release (%)			
	Control	10	30	100
2	31.2 ± 3.11	27.4 ± 4.96	14.9 ± 2.97 **	18.2 ± 1.35
4	36.6 ± 2.67	30.2 ± 4.57	17.4 ± 7.24 **	14.0 ± 5.14

* Concentration (μg/mL).

** $p < 0.05$ compared with the control group (Dunnett's test).

3. EXPERIMENTAL METHODS

^{1}H and ^{13}C NMR spectra were obtained on a JEOL JNM-EX 400 spectrometer in $CDCl_3$ operating at 400 and 100 MHz respectively, with Me4Si as internal standard. IR spectra were recorded with Shimadzu FTIR-8100A spectrometer. Melting points were measured in open capillary tubes and are uncorrected. Gas liquid chromatography and gas chromatograph-mass spectroscopy were carried out with Hewlett Packard HP 6890 GC and Hewlett Packard HP 5972 MSD, equipped with capillary column (TC-1, id 0.25 mm × 30 m, I.D. 1.0 μm), respectively. The conditions of GC-MS were column temperature at 50 ℃ for 5 min then to 270 ℃ at 3 ℃/min, injection temperature at 240 ℃, interface temperature at 230 ℃, ionization voltage 1.3 kV, He as carrier gas, flow rate at 2 mL/min. And the structures were estimated through Library NIST (National Institute of Standards and Technology) WebBook.

3.1. Solvent fractionation and activity tests

Chinese lichens (283 g) were collected during May 2008 at Zhangjiajie, Hunan province, China. Dried lichens were ground and extracted with methanol (3.0 L) at room temperature (20 ± 2 ℃) for 1 month. After filtering, the methanol extract (400 mL) was evaporated *in vacuo* to give a brown oily residue (3.0 g).

The oily residue (3.0 g) was sequentially extracted with hexane, diethyl ether, and ethyl acetate to obtain 0.681, 0.788, and 0.037 g of oily extracts, respectively. The diethyl ether extract (0.770 g) was separated on silica gel using hexane-ethyl acetate (1:9) to afford four fractions: A-1 (0.237 g), A-2 (0.041 g), A-3 (0.021 g), and A-4 (0.146 g). Fraction A-4 (0.1 g) was chromatographed using preparative thin layer chromatography (TLC, silica gel 60, hexane-ethyl acetate (3:2)) to give five fractions A4-1 (R_f = 0.76,

0.014 g), A4-2 (R_f = 0.55, 0.052 g), A4-3 (R_f = 0.45, 0.020 g), A4-4 (R_f = 0.39, 0.010 g), and A4-5 (R_f = 0.27, 0.028 g).

3.2. 3,5-Dihydroxytoluene (Orcinol) (**1**)

Fraction (A4-1) was re-chromatographed using preparative TLC (silica gel 60, hexane-ethyl acetate (2:3)) to obtain pure 3,5-Dihydroxytoluene (orcinol) (**1**) as a white crystalline solid.

Yield 0.006 g; m.p. 56–59 ℃ (lit. ℃) [23, 24]; GC-MS *m/z* (%): 124 $[M]^+$ (100 %), 123 (48), 95 (11), 78 (5), 67 (6), 55 (12), 51 (14), 41 (14), 39 (24), 18 (6).

3.3. Methyl 2,4-dihydroxy-6-methylbenzoate (Methyl Orsellinate) (**2**)

Fraction (A4-2) was re-chromatographed using preparative TLC (silica gel 60, hexane-ethyl acetate (1:1)) to obtain pure methyl 2,4-dihydroxy-6-methylbenzoate (methyl orsellinate) (**2**) as a white crystalline solid.

Yield 0.040 g; m.p. 139–143 ℃ (lit. ℃) [24, 25]; IR (KBr) 3370 (OH), 3316 (OH), 1651 (C=O), 1616 (C=C) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.49 (3H, s, CH_3), 3.93 (3H, s, OCH_3), 5.37 (1H, s, OH), 6.24 (1H, s, ArH), 6.29 (1H, s, ArH), 11.79 (1H, s, OH); ^{13}C NMR ($CDCl_3$) δ 24.25, 51.78, 100.97, 105.36, 110.99, 143.62, 159.76, 164.86, 171.66; MS *m/z* (%): 182 $[M]^+$, 150 (100), 122 (63), 94 (20), 69 (30), 66 (27), 53 (25).

3.4. Syntheses

3.4.1. Synthesis of methyl 2,4-dihydroxybenzoate (**4**) [26, 27]

A solution of 2,4-dihydroxybenzoate (**3**) (1.54 g, 10 mmol) in methanol (50 mL) containing sulfuric acid (10 mL) was refluxed for 24 h at 70 ℃. The reaction mixture was then poured into ice water (100 mL). The mixture was extracted with diethyl ether, washed with saturated aqueous $NaHCO_3$, and dried over anhydrous magnesium sulfate. After evaporation of solvent *in vacuo*, the residue was chromatographed on preparative TLC (diethyl ether-chloroform (1:1)) to give methyl 2,4-dihydroxybenzoate (**4).**

Yield 1.52 g (90.5%); ^1H NMR ($CDCl_3$) δ 3.92 (3H, s, $COOCH_3$), 5.23 (1H, s, OH), 6.37 (2H, d, *J*

= 8.6 Hz, ArH), 7.74 (1H, d, *J* = 8.61 Hz, ArH), 10.97 (1H, s, OH); ^{13}C NMR ($CDCl_3$) δ 51.98, 103.01, 105.85, 107.59, 131.74, 161.53, 163.46, 170.08; MS *m*/*z* (%): 168 $[M]^+$, 137 (35), 136 (100), 108 (44), 95 (6), 81 (12), 80 (14), 69 (15), 53 (22).

3.4.2. Synthesis of methyl 2-hydroxy-4-methoxybenzoate (**5**) [28] and methyl 2,4-dimethoxy-benzoate (**6**)

To a suspension of sodium hydride (60% dispersion in mineral oil, 0.05 g, 1.25 mmol) in THF (5.0 mL) was added a solution of **4** (0.17 g, 1.0 mmol) in THF (10 mL) under an argon atmosphere with stirring for 24 h at room temperature (20 ± 2 ℃). A solution of iodomethane (0.1 g, 0.7 mmol) in THF (3.0 mL) was added to the reaction mixture, which was then continuously stirred for more than 24 h at room temperature. After the reaction mixture was quenched with 2 M HCl solution (5.0 mL), the mixture was extracted with diethyl ether, washed with water and brine, and dried over anhydrous magnesium sulfate. After evaporating of solvent *in vacuo*, the residue was chromatographed on preparative TLC (chloroform-methanol (9:1)) to give compound A (R_f = 0.78) and compound B (R_f = 0.50).

The compound A was re-chromatographed on preparative TLC (hexane-ethyl acetate (8:2)) to give pure methyl 2-hydroxy-4-methoxybenzoate (**5**). The compound B was re-chromatographed using preparative TLC (diethyl ether-chloroform (1:9)) to give pure methyl 2,4-dimethoxybenzoate (**6**).

Yield 0.018 g (9.8%); ^{1}H NMR ($CDCl_3$) δ 3.85 (3H, s, OCH_3), 3.92 (3H, s, $COOCH_3$), 6.44 (2H, d, *J* = 8.18 Hz, ArH), 7.74 (1H, d, *J* = 9.2 Hz, ArH), 10.99 (1H, s, OH); ^{13}C NMR ($CDCl_3$) δ 51.92, 55.41, 100.50, 105.28, 107.43, 131.04, 163.55, 165.37, 170.20; MS *m*/*z* (%): 182 $[M]^+$, 151 (28), 150 (100), 122 (39), 107 (25), 95 (7), 79 (16), 63 (11), 51 (20).

Yield 0.025 g (12.5%); ^{1}H NMR ($CDCl_3$) δ 3.85 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 3.90 (3H, s, $COOCH_3$), 6.50 (2H, d, *J* = 7.8 Hz, ArH), 7.86 (1H, d, *J* = 8.91 Hz, ArH); ^{13}C NMR ($CDCl_3$) δ 51.66, 55.41, 55.90, 98.79, 104.35, 112.05, 133.70, 161.10, 164.03, 165.91; MS *m*/*z* (%): 196 $[M]^+$, 166 (10), 165 (100), 163 (18), 150 (6), 135 (12), 122 (13), 107 (14), 92 (6), 77 (12), 63 (13), 51 (16), 44 (17).

3.4.3. Synthesis of methyl 4-hydroxy-2-methoxybenzoate (**7**) [29]

To a solution of **4** (0.84 g, 5.0 mmol) in dichloromethane (15 mL) was added triethylamine (0.84 g) at

0 ℃ with stirring. A solution of benzoyl chloride (0.77 g, 5.4 mmol) in chloromethane (2.0 mL) was added to the mixture. The reaction mixture was stirred for 24 h at room temperature. After the reaction mixture was quenched with 2 M HCl solution (5.0 mL), the mixture was extracted with chloroform, washed with water, and dried over anhydrous magnesium sulfate. After evaporation of solvent *in vacuo*, the residue was chromatographed on preparative TLC (chloroform) to give methyl 4-benzoyloxy-2-hydroxybenzoate (**4a**) (1.13 g, 83.1%): ^{1}H NMR ($CDCl_3$) δ 3.97(3H, s, $COOCH_3$), 6.80 (1H, dd, *J* = 2.3 and 8.3 Hz, ArH), 6.89 (1H, d, *J* = 2.2 Hz, ArH), 7.53 (2H, t, *J* = 7.7 Hz, ArH), 7.66 (1H, t, *J* = 7.5 Hz, ArH), 7.91 (1H, d, *J* = 8.7 Hz, ArH), 8.19 (2H, d, *J* = 7.1 Hz, ArH), 10.94 (1H, s, OH); MS *m*/*z* (%): 272 $[M]^+$, 105 (100), 77 (35), 51 (18).

To a solution of **4a** (0.66 g, 2.4 mmol) in THF (30 mL) was added KOH (0.44 g, 7.8 mmol). The mixture was stirred for 30 min at room temperature. Methyl sulfate (0.7 mL) was added to the mixture at room temperature and stirred for 12 h. After the reaction mixture was quenched with water and 2 M HCl solution (20 mL), the mixture was extracted with ethyl acetate, washed with brine, and dried over anhydrous magnesium sulfate. After evaporation of solvent *in vacuo*, the residue was chromatographed on preparative TLC (diethyl ether-chloroform (5:95)) to give methyl 4-benzoyloxy-2-methoxybenzoate (**4b**) (0.61 g, 88.6%). ^{1}H NMR ($CDCl_3$) δ 3.87 (3H, s, OCH_3), 3.88 (3H, s, $COOCH_3$), 6.86 (1H, dd, *J* = 2.2 and 8.4 Hz, ArH), 6.89 (1H, d, *J* = 6.2 Hz, ArH), 7.49 (2H, t, *J* = 7.7 Hz, ArH), 7.63 (1H, t, *J* = 7.5 Hz, ArH), 7.90 (1H, d, *J* = 8.4 Hz, ArH), 8.18 (2H, dd, *J* =1.3 and 8.4 Hz, ArH); MS *m*/*z*(%): 286 [M]+, 255 (2), 107 (2), 106 (10), 105 (100), 77 (41), 63 (3), 51 (19).

A mixture of **4b** (0.088 g, 0.31 mmol) and sodium methoxide (0.026 g) in methanol (6.0 mL) was stirred for 12 h at room temperature. After the reaction mixture was quenched with 2 M HCl solution (5.0 mL), the mixture was extracted with ethyl acetate, washed with brine, and dried over anhydrous magnesium sulfate. After evaporation of solvent *in vacuo*, the residue was chromatographed on preparative TLC (hexane-ethyl acetate (2:3)) to give methyl 4-hydroxy-2-methoxybenzoate (**7**) **(**0.041 g, 73.0%). ^{1}H NMR ($CDCl_3$) δ 3.74 (3H, s, OCH_3), 3.86 (3H, s, $COOCH_3$), 6.47 (1H, s, ArH), 6.49 (1H, d, *J* = 2.3 Hz, ArH), 7.61 (1H, s, OH), 7.79 (1H, d, *J* = 8.91 Hz, ArH); ^{13}C NMR ($CDCl_3$) δ 51.80, 55.71, 99.38, 107.16, 111.21, 133.86, 161.14, 161.53, 166.32; MS *m*/*z* (%): 182 $[M]^+$, 152 (8), 151 (100), 149 (19), 136 (6), 121

(13), 108 (17), 93 (10), 80 (7), 65 (16), 52 (15), 51 (15).

3.5. Physiological activity

3.5.1. DPPH-radical scavenging assay [30)]

The evaluation of the DPPH-radical scavenging effect was performed according to the established procedure. Sample compounds were dissolved in ethanol to a concentration of 0.1 mg/mL. A DPPH free radical was dissolved in ethanol to a concentration of 0.2 mM.

3.5.2. Superoxide dismutase (SOD) assay [30)]

SOD-like activity was determined by the nitroblue tetrazolium (NBT) reduction method with a SOD Activity Detection Kit (Wako Pure Chemical Ind. Ltd). Sample compounds were dissolved in DMSO to a concentration of 3.0 mM.

3.5.3. BAP test

The antioxidant activity was determined with a BAP test kit (Uisuma) at 37 ℃. Spectrophotometry (FRAS 4, Uisuma) at 505 nm was used to determine the Fe^{3+} concentration according to a procedure reported in literature [17)].

3.5.4. Cytotoxicity test

Human-derived type-II alveolar epithelial cells (A549) (ATCC: American type cell culture) were used. For cell cultures, the following reagents were obtained from Gibco: fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), phosphate buffer saline (PBS), 0.05%–trypsin-EDTA solution, and DMSO. Cells were passaged and incubated for 24 h, according to a procedure reported in the literature [31)], and stored at room temperature for 0.5 h. 100 μL of Cell Titer-Glo Luminescence reagent (Promega Corporation) were added for the measurement of living cells. Samples were vibrated for 2 min to drive reaction with ATP in the cells, and the number of viable cells was calculated by measuring the color in a luminometer.

3.5.5. Cytokine-production suppression test

The A549 cells (2000 cells/mL), which were subconfluent after the first passage, were placed into 96 well plates and incubated overnight. The stiction of cells was confirmed and the medium was removed. 100 μL of DMEM containing 10% FBS IL-1beta (R & D System Co.) was adjusted to a concentration of 200 μM and added to 100 μL of the test substance (concentrations varied from 1–100 μM). The samples were incubated for 24 h. Finally, the concentration of IL-8 was determined with an ELISA kit according to a procedure reported in literature [32].

3.5.6. Histamine-release assay

Male Wistar rats (7 weeks age, weight 190–220 g, Saitama Experimental Animal Supply Co.) were fed in a room with air-conditioning equipment at room temperature (24 ± 2 ℃) and humidity (45% ± 15%) and were then treated according to procedures in literature [33, 34]. Histamine content in the supernatant was determined by an amino-acid analyzer (Autoanalyzer II, Blanc LOUBET Co., Ltd.).

4. CONCLUSIONS

Methyl 2,4-dihydroxy-6-methylbenzoate (methyl orsellinate) (**2**), which has a high antioxidant activity, was isolated from the Chinese lichen *Gyrophora esculenta* grown in a harsh environment. The physiological activities of **2** and its synthesized related compounds (**4**-**7**) were assessed on the basis of a SOD-like assay, cytotoxicity assay, cytokine inhibitory assay, and histamine-release inhibition assay. In the SOD-like assay, the inhibition rate of **2** (0.1 mg/mL, 11.1%) was higher than that of ascorbic acid (0.1 mg/mL, 12.6%) as a standard antioxidant. Results for the inhibition rates of **4**–**7** indicated that the hydroxyl group at the 4-position played an important role in antioxidative activity. Compound **2**, which exhibited inhibition of active oxygen, showed no cell toxicity, with a cell survival rate of over 80% in a cytotoxicity assay with A549 cells (type II alveolar epithelial cells). In the cytokine inhibitory assay, the family of lichen-based compounds did not show significant inhibition of IL-8 (i.e., inflammatory cytokine) production. In contrast, compounds **2** and **4** exhibited histamine release in rat peritoneal mast cells. Thus, compound **2** exhibited favorable activities in the antioxidant activity assay, cytotoxicity assay, and

histamine-release inhibition assay.

New biological activities of lichen-derived compounds were revealed. Thus, the lichens show potential as a functional food for maintenance and promotion of good health as well as for prevention of lifestyle-related diseases.

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CHAPTER 2. Mango seed oil: the composition of *Mangifera indica* L. seed oil and its cosmetic and pharmaceutical applications

1. INTRODUCTION

Mango (*Mangifera indica* L.) is one of the numerous species of tropical fruit that belong to the *Anacardiaceae* family. Mango originated in North India and the Malay Peninsula. It is cultivated extensively in tropical regions and is grown worldwide. The mango was introduced in Japan early during the Meiji period (1868–1912). In terms of production, about 80% of mangoes are grown in Okinawa and the rest are grown in the Miyazaki, Fukuoka, and Wakayama Prefectures. There are over 500 varieties of mangoes that are selected for specific production areas and local preferences.

Many fragrant functional groups have been identified in mangoes, including alcohols, terpenes, carbonyls, and esters [1-7]. Mangoes contain antioxidants, such as carotenoids, polyphenols, and tannins. Mangiferin is a characteristic component of mango with physiological behavior, such as anti-inflammation [8], anti-diabetic effects [9, 10], immunoregulation [11], antitumor activity [12, 13], and antioxidation [14, 15]; furthermore, mangoes can be used as astringents for mucous inflammation [16-18]. In Brazil, mango leaves have traditionally been used in the treatment of backaches and bronchitis. They have been reported to have analgesic, anti-inflammatory, and immunoregulatory activities [19, 20]. Mango bark has been used in dyes [21, 22]. The seed mainly comprises long-chain fatty acids like oleic acid and stearic acid [23]; this indicates the presence of unsaponifiable matter, such as higher alcohols and sterol. These natural products have not yet been commercialized [24].

Oils are used daily in personal-care products. Plant-based, organic cosmetics are desirable because they are gentle on the skin and have no adverse effects [25-27]. For example, olive extract is a principal ingredient in cosmetics, anti-aging products, and whitening agents [28-30]. Shea butter has a low viscosity and can be absorbed by osmosis; it is often used as a moisturizer in cosmetics because of these beneficial interactions with skin [31-33]. In Japan, commodities related to odor mitigation have increased in popularity: aromatic, antibacterial air fresheners; fabric softeners that transform the smell of sweat; and chewing gum with natural deodorants. Offensive odors have

become increasingly problematic as a result of over 40,000 types of compounds near human living spaces. Nitrogen- and sulfur-containing compounds and low-molecular-weight fatty acids, emanate from sources such as excreta, rotten food (onions, fish), gasoline, and paint thinners [34, 35]. A law was passed in Japan (no. 91, June 1, 1971) to preserve the environment and protect public health by mandating physical deodorization. Numerous products have been developed to reduce the odors of sweat (*n*-butyric acid), musty socks (isovaleric acid), and aging (2-nonenal) [36-38]. These odors are generated by chemical reactions, such as oxidation; consequently, functional compounds in natural foods, such as antioxidants, have been commercialized.

Typical oils, like olive oil, are mainly composed of oleic acid. A case study in Greece has shown an inverse correlation between olive oil intake and the risk of breast cancer [39]. A similar result was reported by the European Prospective Investigation into Cancer and Nutrition (EPIC); the risk of breast cancer in a cohort of postmenopausal women decreased with olive oil intake [40]. A correlation was suggested between decreased breast-cancer risk and the traditional, olive-oil-rich Mediterranean diet. Mechanistically, olive oil may be inhibiting the formation of malignant tumors.

Mango seeds are often discarded as waste after the extraction of mango juice. This study primarily aims to estimate the fatty acid composition of oil extracted from mango seeds; specimens from different cultivation areas were examined. Their potential use in cosmetics was evaluated by producing lotions and soaps, conducting deodorization tests, and analyzing the impact of these results. The secondary aim is to examine the antitumor activity of mango seed oil (MSO) extract. Differential expression proteomics was performed to assess the effects of MSO on HeLa cell growth and examine its safety for therapeutic applications.

2. MATERIALS AND METHODS

2.1. Preparation of mango seed kernel oil

Mango seeds were purchased from Miyazaki, Japan, and Taiwan (2012) from Konan chemical industry (Osaka, Japan). The kernels were dried and ground to a powder using a metallic mill (Waring Laboratory Bleder; Osaka Chemical Co., Ltd.; USA). The powders (Miyazaki, 712.5 g and

Taiwan, 300.8 g) were extracted in *n*-hexane (2,500 mL) for 1 week at room temperature (20 ± 2 ℃). The supernatant was filtered, and the solvent was evaporated *in vacuo*, yielding yellow oil. The mango seeds from Miyazaki and Taiwan comprised 9.78% and 4.02% oil, respectively. The yellow oil was decolorized using activated clay, producing colorless seed oil (Miyazaki, 67.5 g and Taiwan, 11.2 g).

2.2. Determining the composition of fatty acids and lipid classes

Following the conventional method, MSO was esterified using boron trifluoride-methanol (Tokyo Chemical Industry, Japan) to produce volatile methyl esters. The composition of the fatty acids was determined by gas chromatography (GC; GC-2104; Shimadzu) using a TC-70 column (Φ 0.25 mm × 60 m) equipped with a flame ionization detector. The temperatures of the column, injector, and detector were 190 ℃, 250 ℃, and 260 ℃, respectively. Helium was used as the carrier gas.

The methyl ester analogs of the fatty acids were used for peak identification. The retention times were then compared with those of a standard solution of fatty acid methyl esters.

The composition of lipid classes was determined by GC under the same experimental conditions used to characterize fatty acids. A commercial standard was used for peak identification. The retention times were compared with those of a standard solution of glycerol.

2.3. Chemical properties

The neutralization number, unsaponifiable matter, saponification number, acid number, iodine number, and ester value were determined for each MSO sample and measurements were performed in triplicate. These values were determined using conventional methods [41-42].

Neutralization number: MSO (0.25 g) was weighed in triplicate and dissolved in a solution of dimethyl ester and ethanol (25 mL, 1:1).

Unsaponifiable matter: MSO (1.0 g) was dissolved in 1 M solution of potassium hydroxide (KOH) in ethanol (10 mL) and refluxed for 1 h.

Saponification number: MSO (0.8 g) was dissolved in 0.5 M solution of potassium hydroxide in ethanol (25 mL) and refluxed for 1 h.

Acid number: MSO (2.0 g) was dissolved in a solution of dimethyl ester and ethanol (20 mL, 2:1).

Ester value: the ester value was calculated as the saponification number minus the acid number.

Iodine number: MSO (0.3 g) was dissolved in cyclohexane (10 mL). Iodine monochloride solution (Wijs' solution, 25 mL) was added, and the solution was kept in the dark for 30 min at room temperature (18–20 ℃).

2.4. Deodorizing effect

2.4.1. Preparation of odorous substances

Chemicals with offensive odors were purchased from Kishida Chemical Co. (Osaka, Japan): aqueous ammonia (28%), trimethylamine, methyl mercaptan, acetic acid, formaldehyde (37%), acetaldehyde, isovaleric acid, and allyl mercaptan; *trans*-2-nonenal was purchased from Sigma-Aldrich (Tokyo, Japan). To prepare a hydrogen disulfide solution, sodium sulfide nonahydrate and hydrochloric acid (35%) were purchased from Kishida Chemical Co. (Osaka, Japan).

2.4.2. Determination of the deodorization rate of MSO with a gas detector tube [43, 44)]

Six odorous solutions were diluted with deionized water to obtain the correct concentrations: 0.5% aqueous ammonia, 0.3% trimethylamine, 10% hydrogen disulfide, 3% acetic acid, 3% isovaleric acid, and 5% acetaldehyde. Methyl mercaptan was diluted three-fold with benzene, and formaldehyde was diluted 300-fold with deionized water. Hydrogen disulfide was mixed with a 10% solution of sodium sulfide and hydrochloric acid (diluted 20-fold with deionized water). These were used as the eight standard solutions.

The initial concentrations of the standard solutions were adjusted to ammonia (150 ppm),

trimethylamine (20 ppm), hydrogen disulfide (20 ppm), methyl mercaptan (5 ppm), acetic acid (50 ppm), isovaleric acid (50 ppm), formaldehyde (30 ppm), and acetaldehyde (100 ppm).

Each standard solution was placed in a flask and charged with 1 g of MSO. The flask was sealed (airtight) for 30 min at room temperature (20 ± 2 ℃). Thereafter, the concentration of the odorous substance was determined by a Kitagawa AP-20 gas detector (Komyo Rikagaku Kogyo, Kawasaki, Japan) with a Kitagawa gas detector tube. A 100-mL sample of the headspace gas was analyzed. A control test was performed without fatty acids.

The deodorizing rate (%) was calculated according to the following equation:

$$\text{Deodorizing rate (\%)} = \frac{\text{Control value} - \text{Sample value}}{\text{Control area value}}$$

2.4.3. Determination of the deodorizing rate of MSO with a flame ionization detector (FID) [45, 46)]

The concentration of a 0.2% solution of *trans*-2-nonenal was adjusted with ethanol. The concentration was determined by GC (GC-2014AF, Shimadzu) equipped with an FID (200 ℃) and a Unisole F-200 30/60 column (Φ 3.2 mm × 2.1 m). The column temperature was maintained at 120 ℃. Helium (50 mL/min) was used as a carrier gas.

Furthermore, 1 g of MSO was placed in a flask and an adjusted solution of *trans*-2-nonenal (5 μL) was added. The flask was sealed (airtight) for 30 min at room temperature. A 2-mL sample of headspace gas was used for analysis. A control test was performed without fatty acids.

The deodorizing rate (%) was calculated according to the following equation:

$$\text{Deodorizing rate (\%)} = \frac{\text{Control area value} - \text{Sample area value}}{\text{Control area value}}$$

2.4.4. Determination of the deodorizing rate of MSO with a flame photometric detector (FPD) [47, 48)]

A 0.001% solution of allyl mercaptan was prepared in ethanol. 1 g of MSO was placed in a flask and an adjusted solution of allyl mercaptan (10 μL) was added. Then, the flask was sealed

(airtight) for 30 min at room temperature. A 2-mL sample of the headspace gas was used for analysis. A control test was performed without fatty acids.

The remaining concentration of ally mercaptan compound was determined by a GC (GC-2014AF, Shimadzu) equipped with an FPD (200 ℃) and a 1,2,3-tris(2-cyanoethoxy)propane column (Φ 3.2 mm × 2.1 m). The column temperature was maintained at 50 ℃. Helium (40 mL/min) was used as a carrier gas.

The deodorizing rate (%) was calculated according to the following equation:

$$\text{Deodorizing rate (\%)} = \frac{\text{Control area value} - \text{Sample area value}}{\text{Control area value}}$$

2.5. Experimental production of cosmetics

2.5.1. Experimental production of soap

Sodium hydroxide (2.36 g) was dissolved in purified water (0.88 mL) and kept isothermal at 40 ℃. MSO (6.76 g) was heated and added dropwise to the NaOH solution [28-29]. The pH of the MSO soap dissolved in water was determined.

2.5.2. Foaming and anti-foam testing of soaps

The foaming of MSO soap, olive oil soap, and commercial soap (Cow Brand Soap Blue Box, Cow Brand Soap Kyoshisha Co., Ltd.) were compared. 15 mg of soap was added to a graduated cylinder followed by warm water (2 mL, 40 ℃). The cylinder was shaken vigorously 400 times, and the height of the foam was measured immediately and after 1 to 7 h.

2.5.3. Detergency of soap

The detergency of MSO-soap was tested lipstick and sauce stains on clothing. The soap was applied to the stain and scrubbed 20 times using a toothbrush [30].

2.5.4. Experimental production of lotion

MSO (4.50 g) was added to vegetable emulsifying wax (1.65 g) and dissolved in hot water at 60–70 ℃. Purified water was added drop wise, and the solution was mixed for 20 min.

2.6. Differential expression proteomics

2.6.1. Cell culture

HeLa cells derived from human cervical cancer cells (DS Pharma Biomedical, Japan) were used in this study. The cells were grown at 37 ℃ in a 5% CO_2 atmosphere; the culture dishes comprised Dulbecco's Modified Eagle Medium (DMEM, Nihon Pharmaceutical, Japan), 10% (v/v) fetal bovine serum (FBS, Biowest, USA), 50 U/mL penicillin–streptomycin (Invitrogen, USA), 75% $NaHCO_3$, and L-glutamine. Cells were passaged using a trypsin–EDTA solution (0.05%–0.02%) every 2 days.

2.6.2. Water-soluble tetrazolium salt-1 (WST-1) assay

HeLa cells (at concentration of 0.5×10^5 and 0.25×10^5 cells/mL) were added into each well of a 96-well plate and then incubated for 24 h. Dimethyl sulfoxide (DMSO) was dissolved in MSO at a DMSO concentration of 1% (v/v). Next, cells were exposed to concentrations of MSO ranging from 0–500 μg/mL and incubated for 24 h. After treatment, premixed WST-1 (Takara Bio., Japan) was added to each well and incubated for 3 h at 37 ℃. The absorbance was measured at 450 nm using a spectrophotometer (Molecular Devices Co.).

2.6.3. Proteomic analysis

HeLa cells (6.36×10^4 cells/cm^2) in DMEM/10%-FBS were added to a 10-cm dish and incubated at 37 °C for 24 h. The final concentration of MSO was adjusted to 75 μg/mL with DMSO; it was added to the cells and incubated at 37 ℃ for 24 h. DMSO was used as a control. The medium was removed and washed twice with phosphate buffer saline (PBS).

2.6.3.1. Protein extraction

The cells (control and MSO-treated) were collected and charged with 500-mM triethylammonium bicarbonate (TEAB, Sigma, Tokyo, Japan) containing 7 M urea and 0.1%-NP-40. The solids were dissolved using sonication and the solution was rotated overnight in a cold room. After centrifugation, the eluate was exchanged with 50-mM TEAB using spin concentrators (Corning, Tokyo, Japan). The protein concentration was determined using a bicinchoninic acid protein assay (Thermo Fisher Scientific, Rockford, IL, USA).

2.6.3.2. iTRAQ labeling

iTRAQ labeling was performed according to the method reported by Matsumoto [49]. Each sample was denatured, reduced, and digested by trypsin (AB Sciex). Each digest was labeled with a different iTRAQ tag using an iTRAQ Reagent Multiplex kit (AB Sciex). iTRAQ label 116 was used for labeling control samples, and iTRAQ label 117 was used for MSO-treated samples. The labeled samples were combined and fractionated into six fractions using strong cation exchange (SCX) chromatography, according to the manufacturer's instructions (AB Sciex).

2.6.3.3. Analysis by chromatography and mass spectroscopy

Chromatography and spectroscopy measurements were performed based on the methods reported by Matsumoto [50]. One fraction from SCX chromatography was fractionated to 171 spots with a liquid chromatography (LC) system (DiNa nanoLC, KYA Technologies Co., Tokyo, Japan). Each fraction was diluted in a matrix (4 mg/mL α-cyano-4-hydroxycinnamic acid, Wako Pure Chemical Industries Ltd., Osaka, Japan). Peptide samples were analyzed on a matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (MS, 5800 MALDI-TOF/TOF MS/MS) with TOF/TOF series software (AB Sciex). MS/MS data was analyzed by ProteinPliot ^{TM} software (AB Sciex). A statistical analysis was performed to compare the control and MSO-treated samples; $p < 0.05$ was considered statistically significant.

2.6.3.4. Bioinformatic analysis

Panther software was used for the protein-classification analysis.

2.6.3.5. Statistics

The results of WST-1 assay were expressed as the mean ± the standard error of the mean. Statistical analysis was performed using an ANOVA and Dunnett's test. Results with $p < 0.05$ were considered statistically significant. Statistical tests were performed using EZR (Satitama Medical Center, Jichi Medical University, Saitama, Japan).

3. RESULTS AND DISCUSSION

3.1. Composition of lipid classes and fatty acids

GC analysis revealed that triacyl glycerol was the main type of glycerol present in MSOs from both Miyazaki and Taiwan (Table 10). The main fatty acids in MSO were palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), and linolenic acid ($C_{18:3}$) (Table 11). For comparison, mangoes from Thailand, the Philippines, and Mexico were purchased from a market in Japan; seed oil was extracted from these mangoes to determine the fatty acid composition. The major fatty acids determined experimentally agreed with the findings of Muchiri [23]: stearic acid (Thailand, 42.2%; the Philippines, 57.5%; and Mexico, 34.2%) and oleic acid (Thailand, 44.8%; the Philippines, 34.3%; and Mexico, 55.4%). The other fatty acids were palmitic acid (Thailand, 5.8%; the Philippines, 4.7%; and Mexico, 5.9%) and linoleic acid (Thailand, 4.5%; the Philippines, 1.0%; and Mexico, 3.1%). Arachidic acid was absent from the seed oil of mangoes from Miyazaki and Taiwan; it was present in the seed oil of mangoes from Thailand, the Philippines, and Mexico (2.7%, 2.4%, and 1.3%, respectively). The fatty acid composition differed according to the country of production and mango variety. Oleic acid comprised 46% of the fatty acid composition in the Miyazaki MSO but only 44% of the fatty acids in the Taiwan MSO. In contrast, the seed oil from Taiwan mangoes had a slightly higher amount of the other fatty acids as compared with the Miyazaki MSO. The fatty acid composition of MSO is nearly the same as that of shea butter, which is widely used in cosmetics. Therefore, MSO might find applications in cosmetics.

Table 10 Lipid classes composition and chemical characteristics of mango seed oil.

	Miyazaki	Taiwan
Lipid class composition		
Monoglycerides	0.7	0.3
Diglycerides	2.2	1.4
Triglycerides	93.9	96.6
Free fatty acid	1.7	1.1
Others	1.5	0.6
Chemical properties		
Neutralization number	197.1	—
Unsaponifiable matter (%)	0.016	—
Saponification number	185.6	142.5
Acid number (mg・KOH / g)	3.4	1.4
Ester value	182.2	141.1
Iodine number (g / oil)	45.9	25.4

Table 11 Fatty acid composition of mango seed oil (%).

	Miyazaki	Taiwan	Thailand	Philippines	Mexico
Palmitic acid	6.5	7.1	5.8	4.7	5.9
Stearic acid	39.8	40.1	42.2	57.5	34.2
Oleic acid	46.1	43.7	44.8	34.3	55.4
Linoleic acid	4.7	5.2	4.5	1.0	3.1
Linolenic acid	1.8	2.4	—	—	—
Arachidic acid	—	—	2.7	2.4	1.3
Others	0.9	1.4	—	0.1	0.1

3.2. Chemical properties

The chemical characteristics of seed oil from Miyazaki mangoes were determined; the data are shown in Table 10. The average molecular mass of fatty acids and oils was 284.7 g and 907.0 g, as measured by the neutralization number and saponification number, respectively. The low acidity of MSO indicated that mango seeds were digested by lipases, enabling the direct industrial use of their oil without further neutralization [51]. The unsaponifiable matter was <2%, the ester value was nearly identical to the saponification number, and the free fatty acids were almost completely removed when MSO was decolorized using activated clay. The iodine number of MSO (Miyazaki) was lower than that of olive oil and soybean oil, indicating that it was less susceptible to deterioration.

3.3. Deodorizing effect

Miyazaki MSO was used to deodorize 10 odorous compounds. The oil deodorized 2-nonenal and isovaleric acid by >70%. The former is a main aromatic component in matured beer and soba noodles as well as a cause of human odor, and the latter is principally generated from animal husbandry. MSO deodorized hydrogen disulfide (the cause of the "rotten egg" odor from sludge treatment plants) by ~55% (Fig. 3).

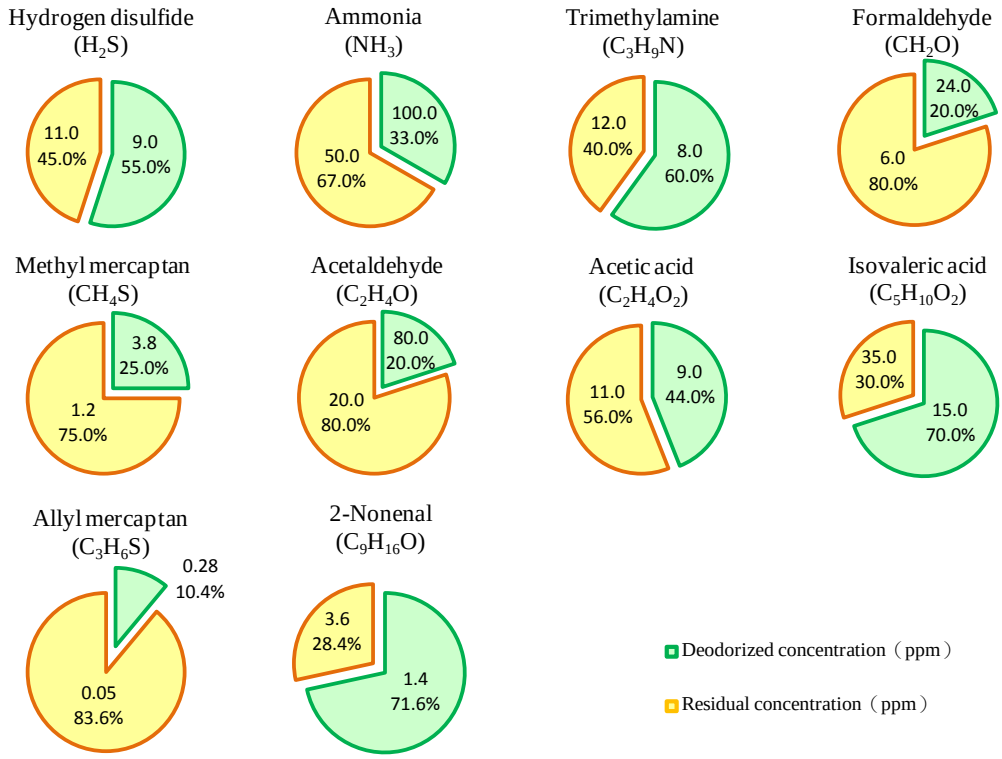


Fig. 3　Deodorizing effect of mango seed oil (Miyazaki).

The deodorizing effect of individual fatty acids in MSO was tested against the same 10 odorous compounds. The most abundant fatty acids were investigated: (A) stearic acid, (B) oleic acid, (C) palmitic acid, (D) linoleic acid, and (E) linolenic acid. Each fatty acid was mixed in several ratios (Table 12) and its effect on the various odorous compounds was quantified; the results are shown in Table 13 and Fig. 4.

Table 12 Mixing ratio of fatty acids.

Fatty acid / Sample No.	(A)	(B)	(C)	(D)	(E)
(1)	1.00 [a)]	-	-	-	-
(2)	-	1.00	-	-	-
(3)	-	-	1.00	-	-
(4)	-	-	-	1.00	-
(5)	-	-	-	-	1.00
(6)	-	0.70	0.10	0.10	0.10
(7)	0.70	-	0.10	0.10	0.10
(8)	0.60	0.30	0.05	0.05	-
(9)	0.30	0.60	0.05	0.05	-
(10)	0.35	0.45	0.05	0.10	0.05
(11)	0.40	0.40	0.10	0.05	0.05
(12)	0.40	0.40	0.10	-	0.10

* A : Stearic acid; B : Oleic acid; C : Palmitic acid; D : Linoleic acid; E : Linolenic acid

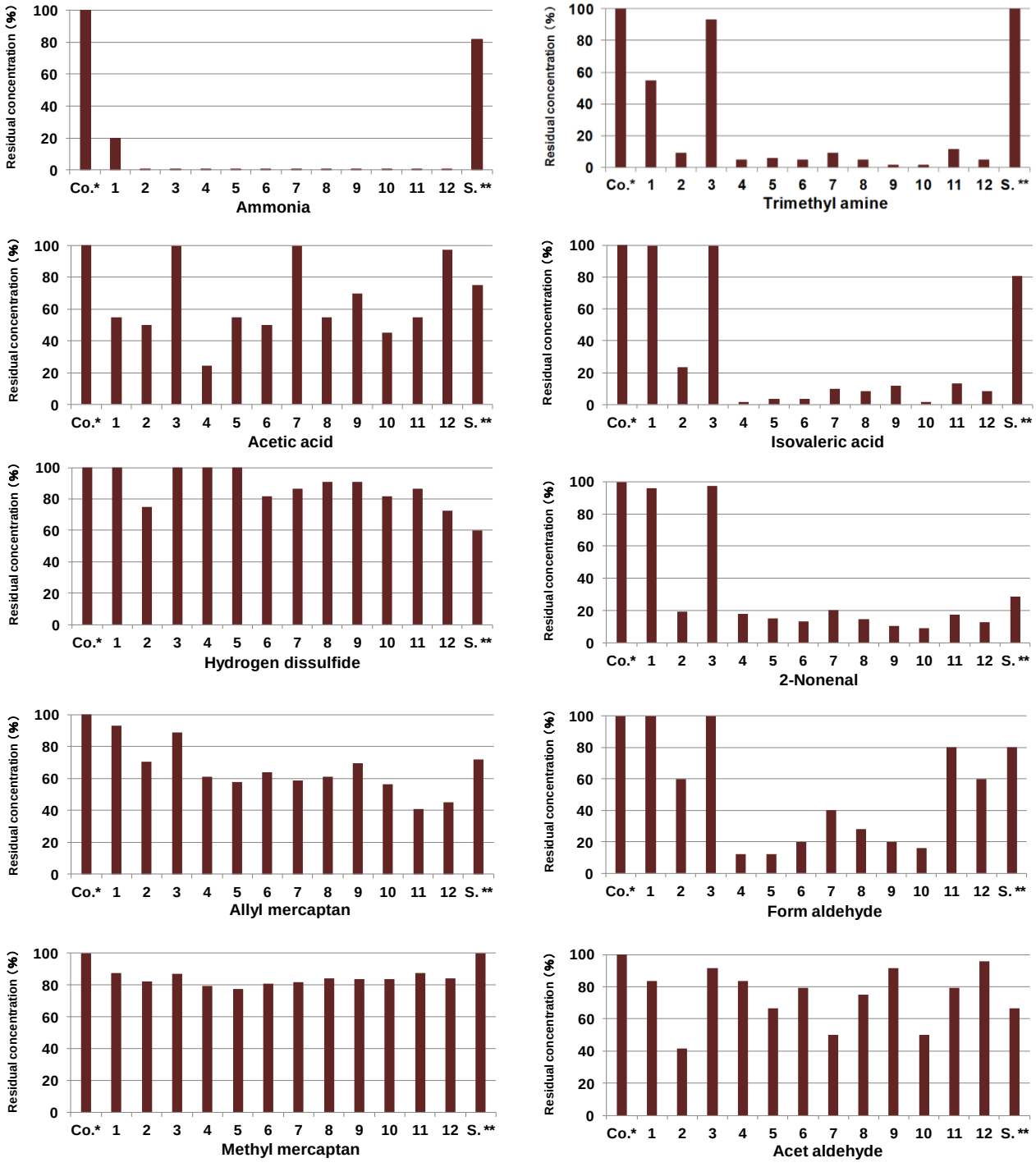
a) Unit : g

Table 13 Deodorizing effect of main fatty acids in mango seed oil.

Sample No.	N		S			Short chain fatty acid		Aldehyde		
	Ammonia	Trimethyl amine	Allyl mercaptan	Methyl mercaptan	Hydrogen disulfide	Acetic acid	Isovaleric acid	Acet aldehyde	Form aldehyde	2-Nonenal
(1)	80.0	45.5	6.9	12.8	0.0	45.0	0.2	16.7	0.0	4.1
(2)	99.7	90.9	29.3	18.0	25.0	50.0	76.7	58.3	40.0	80.7
(3)	99.7	6.8	11.0	13.2	0.0	0.2	0.2	8.3	0.0	2.4
(4)	99.7	95.5	38.8	20.8	0.0	75.5	98.3	16.7	88.0	82.1
(5)	99.7	94.5	42.3	22.4	0.0	45.0	96.7	33.3	88.0	84.8
(6)	99.7	95.5	36.3	19.2	18.2	50.0	96.7	20.8	88.0	86.9
(7)	99.7	90.9	41.1	18.4	13.6	0.2	90.0	50.0	60.0	80.0
(8)	99.7	95.5	39.2	16.0	9.1	45.0	91.7	25.0	72.0	85.2
(9)	99.7	98.5	30.4	16.4	9.1	30.0	88.3	8.3	80.0	89.9
(10)	99.7	98.5	43.7	16.4	18.2	55.0	98.3	50.0	84.0	91.0
(11)	99.7	88.6	59.1	12.4	13.6	45.0	86.7	20.8	20.0	82.4
(12)	99.7	95.5	55.1	16.0	27.3	2.5	91.7	4.2	40.0	87.5
Shea butter	18.2	0.0	28.1	0.0	40.0	25.0	19.2	33.3	20.0	71.2

*Above 70% have good deodorizing effect

Ammonia, mainly produced by farming and drying chicken manure, was deodorized by 99.7% by the individual fatty acids (**1**)–(**5**). Fatty acids (**2**), (**4**), and (**5**) were effective deodorants for trimethylamine, a compound that is widely distributed in nature and originates from aquarium fish, decorative plants, and rotting crustaceans. Fatty acids (**4**) and (**5**) deodorized isovaleric acid by 96%–98% and deodorized 2-nonenal and formaldehyde (which is used as a component in sterilizing agents, antiseptics, and synthetic resins) by 82%–88%. Deodorizing effects beyond those mentioned were not observed.



*Control, **Shea

Fig. 4 Deodorizing effect against 10 odorous substances of main fatty acids in mango seed oil and their mixtures.

These results suggest a synergistic effect between combinations of the main fatty acids in MSO. The deodorizing effect of specified fatty acid combinations was investigated. Nearly all combinations (**6**)–(**12**) deodorized ammonia by ~100%. A combination of (**9**) and (**10**), wherein (B) was the principal ingredient, deodorized trimethylamine by >98%. A combination of (**6**) and (**10**) significantly deodorized isovaleric acid; in particular, (**10**) contained 10% linolenic acid and exhibited a deodorizing efficiency >98%. When the ratio of linoleic acid:linolenic acid was increased in (**10**), it deodorized 2-nonenal by >90%.

The proportion of linoleic acid strongly influenced the deodorizing effect of a fatty acid mixture. Fatty acid combination (**10**) was effective against formaldehyde. A mixture of (C), (D), and (E) exhibited reduced deodorization against allyl mercaptan than a mixture of only (C) and (D). From these results, it was shown that (A) and (C) did not have much influence on the deodorizing effect. Deodorizing effects beyond those mentioned were not observed.

The common methods of deodorization are chemical (chemical reactions), physical (absorption or adsorption), biological (suppressing microorganisms), and masking (perfume components) [52, 53]. An example of chemical deodorization is when an odorous compound is neutralized by fatty acids, yielding an odorless product (the combinations of odorless substances were not determined). Specifically, fatty acids contain –C=C– or –CO– groups that form free radicals and scavenge odorous compounds. Masking occurs when an odorous compound is present below its olfactory threshold. Mixtures of fatty acids have a high vapor pressure (vapor concentration); consequently, the vapor pressure of an odorous compound dissolved in the mixture is reduced. When the vapor concentration of an odorous compound is less than its olfactory threshold, the odor is not detected.

3.4. Experimental production of lotion and soap

The seed oil from Miyazaki mangoes was used to prepare lotion and soap because of its strong deodorizing effect on 2-nonenal and isovaleric acid. Lotions prepared using MSO and olive oil were compared with a commercial product. The MSO lotion was a viscous liquid and with a

smooth texture similar to moisturizing cream, as suggested by 10 volunteer subjects. Regular dermal application of the MSO lotion revealed that its moisturizing capabilities exceeded the other lotions tested. The major components of MSO, stearic acid, palmitic acid, and linoleic acid, are occlusive ingredients used in commercial moisturizing products [54-55]. The pH of MSO soap (pH 8.5) was almost identical to that of a commercial soap (pH 8.7). MSO is a suitable candidate for use in cosmetics.

3.4.1. Evaluation of foam, foam stability, and detergency

The foaming properties of the MSO soap were tested and compared with those of an olive oil soap and a commercial soap. The foam height of the olive oil soap, MSO soap, and the commercial soap were 6.5 cm, 5.2 cm, and 4.4 cm, respectively. The MSO soap foamed well, but the commercial soap produced the thickest foam. The stability of the foam was evaluated by measuring the height of foam over time. The commercial soap exhibited stable foam, whereas the foam formed from the MSO soap dissipated completely after 7 h. Foam from the olive oil soap dissipated completely after 5 h (Fig. 5).

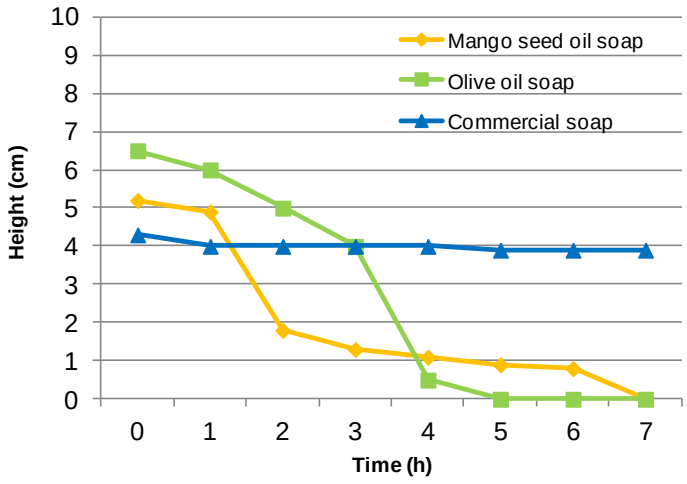


Fig. 5 Anti-foam testing for mango seed oil soap.

The detergency of the soap was determined by visual observation of the removal of lipstick and sauce stains from fabric (Fig. 6). The qualitative evaluation of the washing effect was determined following the general washing procedures. The MSO soap exhibited the greatest detergency, possibly because its principal components are stearic and oleic acid.

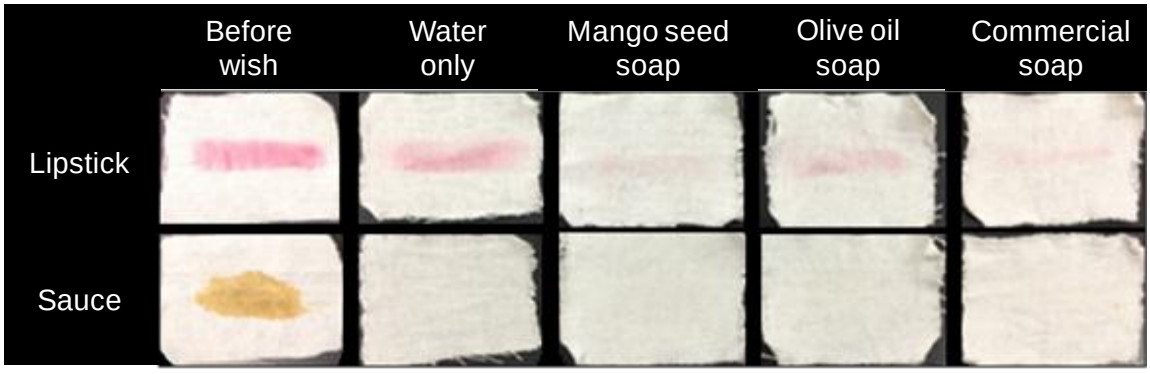


Fig. 6 Evaluation of washing effect of mango seed oil by visual observation method.

3.5. Proteomics analysis

Experiments were conducted to examine the effect of MSO on the viability of HeLa cells. Cell densities of 0.25×10^5 and 0.5×10^5 cells/mL were exposed to varying concentrations of MSO. HeLa cells at a density of 0.25×10^5 cells/mL exhibited proliferative activity that depended on the concentration of MSO: the proliferative activity of cells significantly increased at MSO concentrations of 75 and 100 μg/mL but decreased at the highest MSO concentration, 500 μg/mL (Fig. 7). The proliferation of cells at a density of 0.5×10^5 cells/mL activity did not vary with the concentration of MSO; however, MSO increased the viability of the cells.

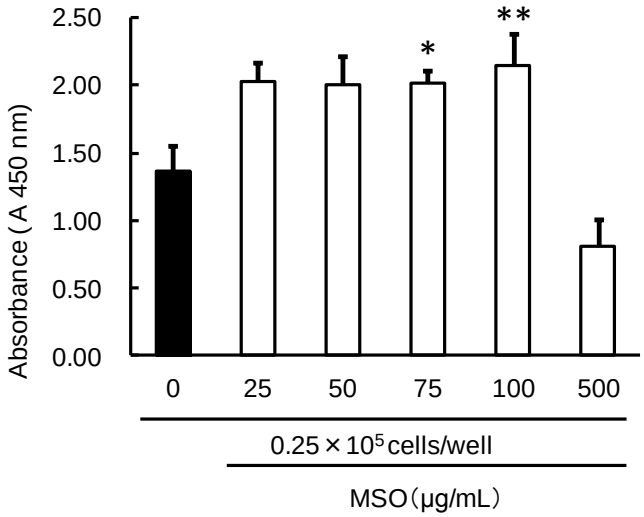


Fig.7 Evaluation of HeLa cell proliferation by WST-1assay. HeLa cells were exposed to MSO (25-500 μg/mL) for 24 h. Data are representative of 3 separate experiments. * : $p<0.05$ and ** : $p<0.01$ are compared with the control (0 μg/mL).

Lipids are a basic nutrient in biological systems and also serve various physiological functions. These physiological functions depend on the composition of fatty acids [56)]. MSO comprises more

than 90% triglycerides: 47.2% saturated fatty acids, 46.3% monounsaturated fatty acids, and 6.5% ω-6 fatty acids. Two potential mechanisms explain the effects of MSO on cell proliferation: either the triglyceride acts directly or the lipoprotein lipase resolves MSO into a fatty acid, which then acts on the cell. Fatty acids have a positive impact on cell proliferation. It has been reported that saturated fatty acids can induce apoptosis in HepG2 cells [57)] and suppress apoptosis induced by monounsaturated fatty acids [58)]. Thus, the various triglycerides and fatty acids in MSO may exhibit additive effects on the proliferation activity of HeLa cells.

Oleic acid has been shown to enhance the proliferation of normal human epithelial cells [59)]. Either oleic or linoleic acid may also encourage the production of vascular endothelial growth factor (VEGF), contributing to wound healing [60)]. Either oleic or linoleic acid transiently promoted cell proliferation during the wound healing process in rat models, leading to shorter healing times [53)]. MSO may have similar effects on cell proliferation and wound healing.

Differential expression proteomics analysis was performed using iTRAQ reagent in two replicates. The expression of 73 proteins, detected in both samples, was identified ($p < 0.05$): 44 proteins significantly increased (≥1.3-fold), 7 proteins significantly decreased (<0.77-fold), and 22 proteins changed between 0.77- and 1.3-fold (Tables 14, 15, and 16).

HMGN2 exhibited the most significant increase in expression, a 25.06-fold increase compared with the control. HMGN2 is a non-histone protein of the high mobility group N (HMGN) family; it specifically binds to nucleosomes, changing the structure of chromatin and thus controlling the complex formation of various transcription factors in the promoter–enhancer region [61)].

HMGN1, HMGN2, and HMGN4 proteins are universally expressed in cells. HMGN1 inhibits DNA repair and cancer progression [62)], whereas increased levels of HMGN5 were discovered in highly metastatic breast cancer cells, indicating an association with enhanced metastatic potential. This shows that proteins in the same family can have reciprocal effects on the same physiological processes [63)]. In this study, the increased expression of HMGN2 likely contributed to the functional

Table 14 List of differentially expressed protein in HeLa cells induced with MSO (Up-regulated).

Unused ProtScore [a]	Peptides (95%) [b]	UniProt number	Gene Symbol	Protein name	iTRAQ ratio Average [c]
2.0	2	P05204	HMGN2	Non-histone chromosomal protein HMG-17	25.06
2.5	1	P07195	LDHB	L-lactate dehydrogenase B chain	5.17
14.6	17	P80723	BASP1	Brain acid soluble protein 1	3.85
8.5	6	P27797	CALR	Calreticulin	3.65
5.0	3	P23528	CFL1	Cofilin-1	3.48
4.1	2	P08195	SLC3A2	4F2 cell-surface antigen heavy chain	3.13
9.4	4	P16403	HIST1H1C	Histone H1.2	3.06
5.6	2	P55072	VCP	Transitional endoplasmic reticulum ATPase	2.67
6.2	4	P04792	HSPB1	Heat shock protein beta-1	2.66
7.5	4	Q71DI3	HIST2H3A	Histone H3.2	2.56
2.0	1	P62906	RPL10A	60S ribosomal protein L10a	2.43
23.0	20	P10809	HSPD1	60 kDa heat shock protein, mitochondrial	2.40
7.9	4	P61604	HSPE1	10 kDa heat shock protein, mitochondrial	2.40
2.0	1	P50402	EMD	Emerin	2.35
14.7	9	P35579	MYH9	Myosin-9	2.28
2.6	1	Q16881	TXNRD1	Isoform VI of Thioredoxin reductase 1, cytoplasmic	2.18
7.9	6	P63104	YWHAZ	14-3-3 protein zeta/delta	2.00
22.8	15	P08238	HSP90AB1	Heat shock protein HSP 90-beta	1.90
6.2	4	P10412	HIST1H1E	Histone H1.4	1.90
10.4	6	P62937	PPLA	Peptidyl-prolyl cis-trans isomerase A	1.85
21.4	17	P08670	VIM	Vimentin	1.82
8.1	5	P00558	PGK1	Phosphoglycerate kinase 1	1.78
4.0	2	P62826	RAN	GTP-binding nuclear protein Ran	1.76
2.0	1	Q00610	CLTC	Clathrin heavy chain 1	1.74
8.3	8	Q00839	HNRNPU	Heterogeneous nuclear ribonucleoprotein U	1.74
14.0	14	P29401	TKT	Transketolase	1.72
23.2	15	P11021	HSPA5	78 kDa glucose-regulated protein	1.71
12.9	8	Q5VTE0	EEF1AL3	Putative elongation factor 1-alpha-like 3	1.69
3.6	2	P25705	ATP5A1	ATP synthase subunit alpha, mitochondrial	1.63
9.1	4	P06576	ATP5B	ATP synthase subunit beta, mitochondrial	1.56
27.4	23	P05787	KRT8	Keratin, type II cytoskeletal 8	1.56
29.8	18	P14618	PKM2	Pyruvate kinase isozymes M1/M2	1.54
2.0	1	P09382	LGALS1	Galectin-1	1.54
1.5	1	Q15233	NONO	Non-POU domain-containing octamer-binding protein	1.52
8.6	5	P02545	LMNA	Lamin-A/C	1.50
5.2	3	P07237	P4HB	Protein disulfide-isomerase	1.49
8.2	5	P19338	NCL	Nucleolin	1.48
18.9	16	P04406	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	1.48
4.8	3	Q08211	DHX9	ATP-dependent RNA helicase A	1.39
8.9	5	P07737	PFN1	Profilin-1	1.39
18.0	13	P06733	ENO1	Alpha-enolase	1.39
24.1	13	P08729	KRT7	Keratin, type II cytoskeletal 7	1.36
10.2	7	Q06830	PRDX1	Peroxiredoxin-1	1.34
2.2	1	P23246	SEPQ	Splicing factor, proline- and glutamine-rich	1.34

a) A score of protein confidence (ProtScore) for a detected protein that is calculated from the peptide confidence from spectra that are not already "used" by higher scoring proteins in the experiments.

b) The number of distinct peptides with at least 95% confidence in the experiments.

c) Average of iTRAQ ratios measured by duplicated experiments.

Table 15 List of differentially expressed protein in HeLa cells induced with MSO (Continued).

Unused ProtScore [a)]	Peptides (95%) [b)]	UniProt number	Gene Symbol	Protein name	iTRAQ ratio Average [c)]
2.3	1	Q99497	PARK7	Protein DJ-1	1.28
2.1	1	P50454	SERPINH1	Serpin H1	1.27
38.1	24	P21333	FLNA	Filamin-A	1.27
10.6	7	P38646	HSPA9	Stress-70 protein, mitochondrial	1.26
10.2	6	P13639	EEF2	Elongation factor 2	1.25
4.5	2	P14625	HSP90B1	Endoplasmin	1.23
10.0	8	P31943	HNRNPH1	Heterogeneous nuclear ribonucleoprotein H	1.23
18.2	16	Q04695	KRT17	Keratin, type I cytoskeletal 17	1.14
3.5	2	P53396	ACLY	ATP-citrate synthase	1.10
2.0	1	P62857	RPS28	40S ribosomal protein S28	1.07
4.1	2	P49327	FASN	Fatty acid synthase	1.04
3.0	1	O43707	ACTN4	Alpha-actinin-4	0.99
2.3	1	P49411	TUFM	Elongation factor Tu, mitochondrial	0.95
3.4	2	P18124	RPL7	60S ribosomal protein L7	0.95
4.9	3	P00338	LDHA	L-lactate dehydrogenase A chain	0.95
4.6	2	P36578	RPL4	60S ribosomal protein L4	0.93
5.7	4	P40926	MDH2	Malate dehydrogenase, mitochondrial	0.90
6.0	3	P60174	TPI1	Triosephosphate isomerase	0.89
2.9	2	P06748	NPM1	Nucleophosmin	0.88
2.4	1	P22314	UBA1	Ubiquitin-like modifier-activating enzyme 1	0.78
9.5	8	P22626	HNRNPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1	0.78
14.0	9	P30101	PDIA3	Protein disulfide-isomerase A3	0.77

a) A score of protein confidence (ProtScore) for a detected protein that is calculated from the peptide confidence from spectra that are not already "used" by higher scoring proteins in the experiments.

b) The number of distinct peptides with at least 95% confidence in the experiments.

c) Average of iTRAQ ratios measured by duplicated experiments.

Table 16 List of differentially expressed protein in HeLa cells induced with MSO (Down-regulated).

Unused ProtScore [a)]	Peptides (95%) [b)]	UniProt number	Gene Symbol	Protein name	iTRAQ ratio Average [c)]
2.0	1	P39019	RPS19	40S ribosomal protein S19	0.76
2.5	1	P37802	TAGLN2	Transgelin-2	0.76
7.7	5	P62805	HIST1H4A	Histone H4	0.75
3.6	1	O75369	FLNB	Filamin-B	0.69
2.4	1	P84103	SFRS3	Splicing factor, arginine/serine-rich 3	0.63
2.0	1	Q9UI30	TRMT112	tRNA methyltransferase 112 homolog	0.61
4.2	2	P04083	ANXA1	Annexin A1	0.59

a) A score of protein confidence (ProtScore) for a detected protein that is calculated from the peptide confidence from spectra that are not already "used" by higher scoring proteins in the experiments.

b) The number of distinct peptides with at least 95% confidence in the experiments.

c) Average of iTRAQ ratios measured by duplicated experiments.

maintenance of the cell nucleus; while, the cytostatic effect observed in T24 cells and oral squamous cell carcinoma originated from outside the cell [64, 65)].

Annexin (ANXA1), a calcium-dependent phospholipid binding protein, exhibited decreased

expression. Increased expression of this protein has been associated with the incidence and development of breast cancer and decreased cumulative survival rates [66]. Therefore, the cell proliferation effect induced by MSO probably does not enhance the malignancy of cancer cells.

The expression of L-lactate dehydrogenase B chain (LDHB) protein exhibited the second-highest increase. LDHB is an enzyme that catalyzes the conversion of pyruvic acid to lactic acid in the final stages of the glycolysis pathway. MSO may enhance glycolysis (by accelerating the glucose metabolism); this mechanism provides a potential explanation for its proliferative effect on HeLa cells.

The differential expression of proteins was analyzed using Panther software. The results showed changes in expression related to glycolysis, cytoskeletal regulation by Rho GTPase, integrin signaling, Parkinson's disease, ATP synthesis, and pyruvate metabolism pathways (Fig. 8). The glycolysis pathway suffered from significant fluctuations according to the Panther statistical overrepresentation test.

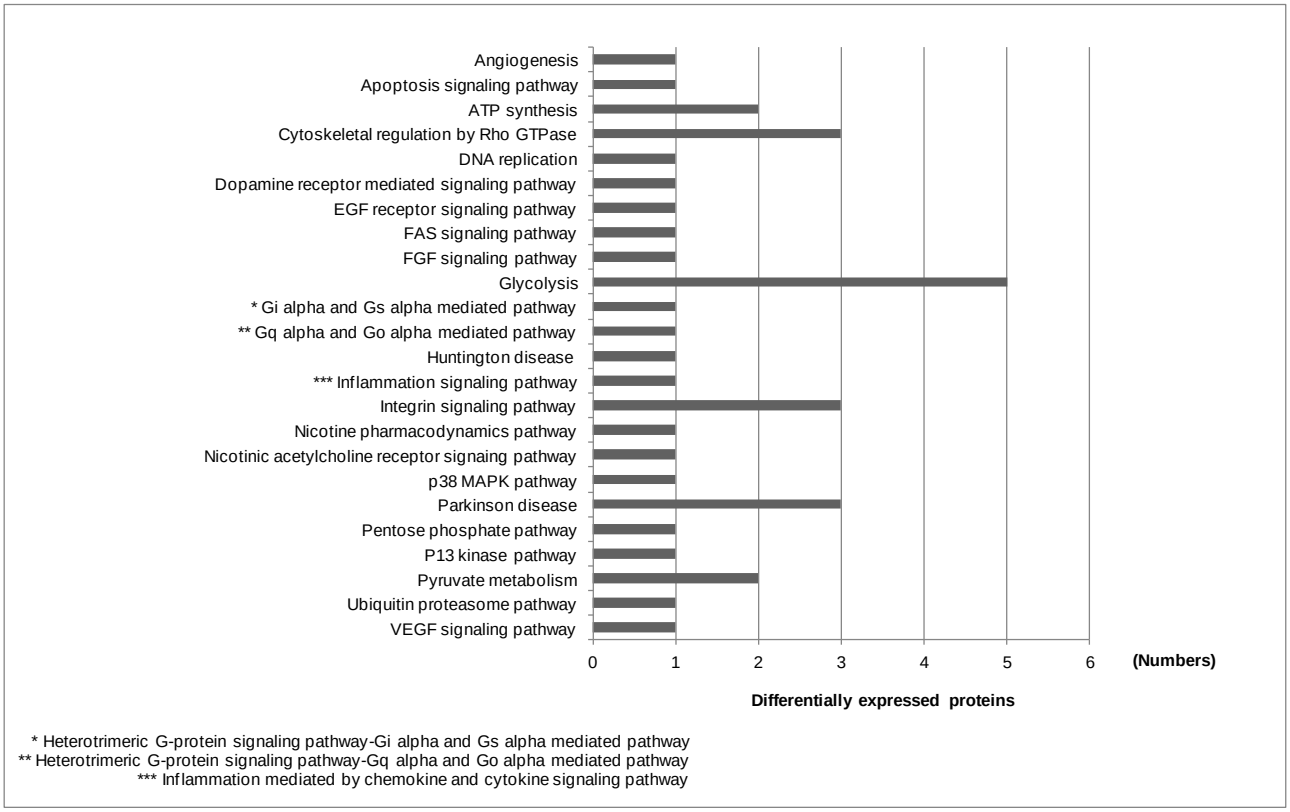


Fig. 8 A graph illustrating the function annotations that were assigned to the filtered list of differentially expressed proteins using Panther software.

Five proteins in the glycolysis pathway were identified: phosphoglycerate kinase 1, pyruvate kinase isozymes M1/M2, glyceraldehyde-3-phosphate dehydrogenase, α-enolase, and triosephosphate isomerase. Except for triosephosphate isomerase, the expression of these proteins was significantly increased by MSO.

It is important to study the metabolism and proliferative activity of cancer cells [67]. In general, cancer cells produce energy by aerobic glycolysis, in contrast to normal cell oxidative phosphorylation. The expression of PKM2 must increase to alter the splicing isoform of pyruvate kinase, which is required during aerobic glycolysis. In this study, proteomic analysis showed that PKM2 increased 1.54-fold compared with the control. The expression of PKM2 was expected to be higher in HeLa cells than in normal cells because the HeLa line is derived from cervical cancer cells; however, MSO caused the expression of PKM2 to increase concomitantly with an increase in HeLa cell proliferation.

MSO caused increased the expression of signaling pathway proteins beyond the expectations for simple cell proliferation. Rho GTPase is involved in the control of the cytoskeleton, the Integrin signaling pathway affects cell adhesion, the ATP signaling pathway affects the synthesis of ATP, and the VEGF signaling pathway affect wound healing [57]. MSO did not significantly change any other pathways, as determined by pathway analysis.

This study shows that MSO increases the proliferative activity of HeLa cells without increasing their malignant potential. The increases in proliferative activity attenuated at an MSO concentration of 500 μg/mL, indicating that any antitumor activity occurs at high concentrations. The individual components of MSO have different effects on cell proliferation.

The mechanism by which MSO stimulates cell proliferation is unknown. In recent years, Briscoe et al. reported that long-chain fatty acids activate G protein-coupled receptor 40 (GPR40) [68]. GPR40 was not only highly expressed in pancreatic islet β cells but also expressed in breast cancer cells and the THP-1 cell line [56, 69, 70]. Although it is unclear whether triglycerides and/or fatty acids directly affect protein expression in HeLa cells, future studies will examine the participating receptor on HeLa cells. It is also important to evaluate the effects of MSO on both

proliferative activity and proteomic analysis in normal tissues, such as fibroblasts or keratinocytes, to determine if MSO affects wound healing.

4. CONCLUSIONS

MSO was extracted and its composition of fatty acids was determined to evaluate its used in cosmetics and new functional materials. The fatty acid composition did not vary largely between mango varieties from Miyazaki and Taiwan; the main components are palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid. The measured iodine number of MSO was low, indicating that it is less susceptible to deterioration.

MSO had the strongest deodorizing effect on 2-nonenal and isovaleric acid. Linoleic acid and linolenic acid, in particular, deodorized isovaleric acid by 96%–98%. Unsaturated fatty acids were shown to correlate with a deodorizing effect. Soaps and lotion were produced from the MSO, and the MSO soap showed good foaming and detergency. These results suggest that MSO is a suitable candidate for many potential applications in the cosmetic industry.

Cell proliferation results by a WST-1 assay showed that MSO has a concentration-dependent effect on the proliferation of HeLa cells. Differential expression proteomics analysis was performed using the iTRAQ reagent and MALDI-TOF/TOF MS/MS; 73 proteins were identified. Compared with a control group, the expression of HMGN2, a non-histone chromosomal protein, increased 25.06-fold; the expression of ANXA1 decreased 0.59-fold. The glycolysis pathway showed significant fluctuations. These results demonstrate that MSO increases the proliferative activity of cells without increasing their malignant potential.

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SUMMARY

Natural, plant-based compounds exhibit various physiological functionalities that can lead to the commercialization of new materials. Investigating the composition and behavior of isolated components enables the design of optimized formulations.

In chapter 1 examines a family of chemicals originating from Chinese lichen. Methyl 2,4-dihydroxy-6-methylbenzoate is an antioxidant that was extracted from plant samples. It was non-toxic to type II A549 cells and inhibited the release of histamine in rat peritoneal mast cells. Similar compounds were synthesized by varying the location and identity of functional groups on an aromatic ring. The biological activity of compounds was related to differences in functional groups.

In chapter 2 investigates the use of MSO for cosmetics and antitumor agents. The main fatty acids in MSO were oleic acid and stearic acid; triacylglycerol was the primary lipid. MSO had a deodorizing effect against 2-nonenal and isovaleric acid, which may relate to unsaturation in the fatty acids. The *in vitro* cytotoxicity of MSO was probed using the WST-1 assay; it significantly promoted HeLa cell proliferation. iTRAQ labeling with MALDI-TOF/TOF MA/MS was used to quantitatively compare the HeLa cell proteomes for MSO-treated and control samples. The expression of HMGN2 increased significantly and that of ANXA1 decreased significantly, and the glycolysis pathway showed significant fluctuations. These findings offer evidence that MSO enhances cell proliferation but not the malignancy of HeLa cells.

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